NEUROGENIN1 and NEUROGENIN2 control two distinct waves of neurogenesis in developing dorsal root ganglia

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Different classes of sensory neurons in dorsal root ganglia (DRG) are generated in two waves: large-diameter trkC$^+$ and trkB$^+$ neurons are born first, followed by small-diameter trkA$^+$ neurons. All such neurons require either *neurogenin* (*ngn*) *1* or *2*, two neuronal determination genes encoding basic helix–loop–helix (bHLH) transcription factors. *ngn2* is required primarily if not exclusively for the generation of trkC$^+$ and trkB$^+$ neurons, whereas the generation of most or all trkA$^+$ neurons requires *ngn1*. Comparison with previous lineage tracing data in the chick suggests that this dichotomy reflects a requirement for the two *ngns* in distinct sensory precursor populations. The neurogenesis defect in *ngn2*$^{-/-}$ embryos is transient and later compensated by *ngn1*-dependent precursors, suggesting that feedback or competitive interactions between these precursors may control the proportion of different neuronal subtypes they normally produce. These data reveal remarkable parallels in the roles of bHLH factors during neurogenesis in the DRG, and myogenesis in the neighboring myotome.

[*Key Words*: Sensory neurons; neurogenesis; neurotrophins; neurogenins; bHLH protein]

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Transcription factors in the basic helix–loop–helix (bHLH) family play a central and phylogenetically conserved role in the determination of cell type. In vertebrates a family of muscle-specific bHLH factors (called the myogenic regulatory factors, or MRFs) is essential for the determination of myoblast fates (for reviews, see Molkentin and Olson 1996; Yun and Wold 1996). Analogously, in *Drosophila* a family of bHLH factors, called proneural genes, is essential for the determination of neural fates (for review, see Anderson and Jan 1997). Strikingly, the cell-type specificity and primary sequence of bHLH factors appear coordinately conserved across phylogeny. Thus, mammalian neurogenic bHLH factors are more highly related to *Drosophila* proneural genes than they are to mammalian myogenic bHLH factors (Johnson et al. 1990), and vice-versa (Michelson et al. 1990).

A puzzling feature of bHLH factors is the apparent multiplication of functionally similar genes expressed within a given tissue. The MRF subfamily, for example, consists of four highly related genes: *myoD*, *myf5*, *myogenin*, and *MRF4/herculin* (Molkentin and Olson 1996; Yun and Wold 1996). Similarly, the *achaete–scute* complex of *Drosophila* contains a tandem array of four highly related proneural genes (Alonso and Cabrera 1988). Some of this multiplication reflects the fact that related bHLH genes act in cascades to control determination and differentiation within both nerve and muscle (Jan and Jan 1993; Weintraub 1993). However, this cannot fully explain the reason for such multiplication, as both loss- and gain-of-function assays have revealed apparent redundancy for genes acting at similar levels in the developmental hierarchy (for reviews, see Weintraub et al. 1991; Campuzano and Modolell 1992).

It has become clear recently that the apparent genetic redundancy of myogenic bHLH factors at the tissue level masks an underlying nonredundant function at the cellular level. The subtle phenotypes of *myf5* and *myoD* single mutants suggested initially that these genes were functionally redundant (Braun et al. 1992; Rudnicki et al. 1992), a conclusion supported by the clear myogenic defects observed in double mutants (Rudnicki et al. 1993). However, more recent analyses have shown that *myf5* and *myoD* are expressed initially by distinct subpopulations of myogenic precursors (Braun and Arnold 1996),

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each of which may compensate for the loss of the other in single mutants (Patapoutian et al. 1995; Braun and Arnold 1996; for reviews, see Molkentin and Olson 1996; Yun and Wold 1996). These data suggested that, at least in muscle, duplication of highly related bHLH determination genes may reflect their utilization by distinct classes of progenitor cells.

The extent to which this mechanism operates more generally is not yet clear. In the vertebrate nervous system, homologs of the *Drosophila* proneural gene *atonal* (Jarman et al. 1993) called *neurogenins/Math4a-c/NeuroD3* have emerged as determination genes for the neuronal fate analogous to *MyoD* and *myf5* (Gradwohl et al. 1996; Ma et al. 1996, 1998; McCormick et al. 1996; Fode et al. 1998). Like the myogenic determination genes, the *neurogenins* (*ngns*) act upstream of a related subfamily of genes exemplified by *neuroD* (Lee et al. 1995), which appear to act as differentiation factors (for reviews, see Kageyama and Nakanishi 1997; Lee 1997). Initial analysis of *ngn1* and *ngn2* single mutants has revealed a block at the earliest stages of neurogenesis, in complementary sets of cranial sensory ganglia (Fode et al. 1998; Ma et al. 1998). However in *ngn1* mutants there is no obvious phenotype in the CNS (Ma et al. 1998), where *ngns* are transcribed in highly overlapping patterns (Gradwohl et al. 1996; Sommer et al. 1996; Ma et al. 1997). This suggests that the *ngns* may act redundantly in some regions of the nervous system.

Such apparent redundancy raises the question of whether the *ngns* function in the same precursor cells, or rather in distinct precursors that can compensate for one another. Here we have addressed this question by examining the roles of the *ngns* in the development of trunk dorsal root ganglia (DRG), which contain several different classes of sensory neurons (Snider 1994; Snider and Wright 1996). We find that most or all small-diameter, nociceptive (trkA$^+$) neurons require *ngn1*, whereas *ngn2* is transiently required only for large-diameter trkB$^+$ and trkC$^+$ neurons. The initial requirement for *ngn2* is, however, subsequently compensated in an *ngn1*-dependent manner. Comparison with previous cell lineage studies in chick DRGs (Frank and Sanes 1991) suggests that *ngn2* and *ngn1* may be required in distinct precursor populations that generate different classes of sensory neurons, analogous to the requirements of *myf5* and *myoD* by distinct subsets of myoblasts. The ability of the *ngn1*-dependent precursors to compensate for the loss of *ngn2*-dependent cells further suggests that feedback or competitive interactions between these two populations may control the production of different classes of sensory neurons during normal development.

Results

Sequential expression of ngn2 *and* ngn1 *in the developing DRG*

To understand better the sensory neuron phenotypes of *ngn1* and *ngn2* single mutants, we first re-examined the expression of the *ngns* during neural crest migration and early dorsal root gangliogenesis in wild-type embryos. The earliest expression of *ngn2* was detected in cells at the lateral margins of the neural tube (Fig. 1B,D,F, arrows). Comparison to the expression pattern of *Hfh2*, a member of the forkhead family of transcription factors identified recently as an early neural crest marker (Labosky and Kaestner 1998), suggested that *ngn2* is expressed by neural crest cells very early in their migration (Fig. 1A,C,E, arrows; cf. Fig. 1F, arrowhead). Expression of *ngn2* continues into the early stages of DRG condensation, and is then extinguished by ~E10.5 (Sommer et al. 1996). In contrast, *ngn1* expression was not detected in newly emigrating crest cells (Fig. 1, F vs. G and H, arrowheads). Rather, *ngn1* expression was detected only after crest cells had migrated to a position between the somite and neural tube and begun their condensation into ganglion primordia (Fig. 1H, arrow).

Additional examination extended the previous observation that expression of *ngn2* and *ngn1* spans two distinct periods in the developing DRG (Sommer et al. 1996) (summarized in Fig. 1I). For example in cervical DRGs, expression of *ngn2* starts at E8.75–E9 (17 somites) and ends at E10.5, whereas expression of *ngn1* starts at E9–E9.25 (20–21 somites) and extends until E13 (data not shown). The onset of *ngn2* expression precedes that of *ngn1* by about four somites of development. However, analysis of *ngn2* mutants (see below) indicates that the earliest expression of *ngn1* likely reflects cross-regulation by NGN2. Therefore the actual onset of NGN2-independent *ngn1* expression follows that of *ngn2* by ~12–18 hr (Fig. 1I, dashed line). From E10.5 to E13, only *ngn1* is expressed in cervical DRG (Fig. 1I).

Two transient but overlapping phases of neuroD *expression revealed by analysis of* ngn1 *and* ngn2 *mutant embryos*

Throughout the rostrocaudal axis, expression of *neuroD* follows that of *ngn2* (Sommer et al. 1996) and spans the period during which *ngn1* expression is initiated and extinguished (Fig. 1I, NeuroD). Analysis of *ngn1* and *ngn2* single mutants revealed, however, that the apparently continuous expression of *neuroD* in developing wild-type DRG (Fig. 2A,C,E,G, +/+) is apparently a composite of two distinct phases of transient expression that follow the rostral-to-caudal gradient of normal DRG development: an early phase (Fig. 1I, green line), which is unmasked in the caudal region of E12 *ngn1*$^{-/-}$ embryos (Fig. 2H, arrow), and a later phase (Fig. 1I, blue line), which is unmasked in the rostral region of E10 *ngn2*$^{-/-}$ embryos (Fig. 2B, arrow). As there is no *neuroD* expression in *ngn1;ngn2* double mutant embryos (C. Fode and F. Guillemot, unpubl.), the early phase must be NGN2-dependent (Fig. 2A,H, arrows), and conversely the later phase is NGN1-dependent (Fig. 2B,G, arrows).

The NGN2- and NGN1-dependent phases of *neuroD* expression were only clearly visible in older (E12) (Fig. 2H) and younger (E10) (Fig. 2B) embryos, respectively. Thus, for example, in *ngn1*$^{-/-}$ embryos younger than E12 the early NGN2-dependent phase has presumably not

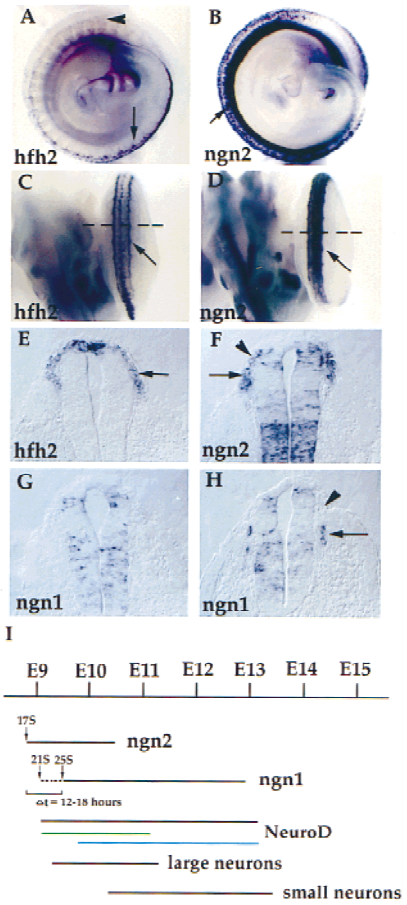

Figure 1. Sequential expression of *ngn1* and *ngn2* in the developing DRG. Whole-mount in situ hybridizations were performed with *Hfh2*, *ngn2*, and *ngn1* as probes. (*A*,*B*) Lateral view of E9.75 (27 somites) embryo. (Arrowhead and arrow in *A*) Rostral and caudal parts of the embryo, respectively. Heads of the embryos are not shown (downward direction). (*C*,*D*) Dorsal view of caudal halves of the same embryos. Expression of *Hfh2* and *ngn2* was detected in emigrating crest cells (*A–D*, arrows), which is clearly seen in transverse sections (*E*,*F*, arrows). Positions of the sections in *E* and *F* are indicated by the broken lines in *C* and *D*. *ngn1* expression was not detected in a section (*G*) at an axial level equivalent to *E* and *F* but was detected in sections through a more rostral part of the embryo (*H*, arrow) (DRG development shows a rostral to caudal gradient). Unlike *ngn2*, *ngn1* was not expressed in newly emigrating crest cells (cf. *H* and *F*, arrowheads). Expression of *ngn2* in cervical DRG was first detected at E8.75–E9 (17 somites) and disappeared at E10.5; expression of *ngn1* started at E9–E9.25 (20–21 somites) and lasted all the way to E13 (data not shown) (summarized in *I*). There is a short period of *ngn2*-dependent *ngn1* expression (*I*, broken line, Δt; see Fig. 6A). Although separate waves of *ngn2*-dependent (green line) and *ngn1*-dependent (blue line) *neuroD* expression can be distinguished clearly in mutants (see Fig. 2B), the end and start points of these two respective waves are approximate. The time line of large and small neuron generation in cervical DRG is adapted from Lawson and Biscoe (1979).

yet been down-regulated, and therefore masks the loss of *neuroD* expression caused by the mutation (Fig. 2C,D, arrowheads). However by E12, down-regulation of NGN2-dependent *neuroD* expression has occurred already in rostral DRG, unmasking the loss of *neuroD* expression in this region caused by deletion of *ngn1* (Fig. 2H, arrowhead; cf. Fig. 2G, arrow). Conversely, in *ngn2*$^{-/-}$ embryos older than E10, the later NGN1-dependent wave has evidently already swept into caudal DRG and masks the loss of *neuroD* expression caused by the mutation (Fig. 2E,F, arrowheads). By contrast, in younger embryos (E10) the later NGN1-dependent phase has not yet been initiated in caudal DRG (Fig. 2B, arrowhead), revealing the loss of *neuroD* expression caused by the *ngn2* mutation (cf. Fig. 2A, arrow vs. 2B). There is clearly some overlap between these two phases, as indicated by the reduced levels of *neuroD* expression in both the NGN2-dependent and NGN1-dependent regions of mutant embryos in comparison with wild type (Fig. 2, cf. A, arrowhead, and B, arrow; cf. G, arrowhead, and H, arrow). Taken together, these data suggest that *neuroD* is expressed during DRG development in two sequential and transient, but overlapping, waves that depend on *ngn2* and *ngn1*, respectively.

Loss of the trkA$^+$ *neurons and a subset of* trkC$^+$ *and* trkB$^+$ *neurons in* ngn1 *mutants*

Neuronal birthdating studies have shown that DRG precursors go through their final divisions in two broad and overlapping successive waves. In the cervical DRG, the first wave, which gives rise to large-diameter neurons, occurs from E9.5 to E11.5, whereas the second wave, which mainly gives rise to small-diameter neurons begins at E10.5 and lasts until E13.5 (Lawson and Biscoe 1979; see Fig. 1I). There is a period of overlap from E10.5 to E11.5 when both sizes of neurons are born concurrently. We noted that the generation of small neurons appears to coincide with the period when only *ngn1* is expressed (Fig. 1I). As the small neurons are nociceptive afferents that primarily express trkA, a receptor for nerve growth factor (NGF) (Mu et al. 1993; Wyatt and Davies 1993; Backstrom et al. 1996), we sought to determine if *ngn1* is required for the development of the *trkA*$^+$ subset of sensory neurons. At E15, the cervical DRG of *ngn1*$^{-/-}$ mutants were much smaller compared with those of wild-type littermates, as indicated by the expression of *SCG10*, a pan-neuronal marker (Stein et al. 1988) (Fig. 3, cf. B and A). This reduction in size was primarily caused by the complete elimination of *trkA*$^+$ neurons (Fig. 3, D vs. C), which constitute up to 70% of total ganglionic neurons (Mu et al. 1993). Loss of the NGF-dependent nociceptive population was further confirmed by a lack of expression of two additional markers in newborn *ngn1*$^{-/-}$ mice: *VR1*, the capsaicin receptor gene (Fig. 3, F vs. E) (Caterina et al. 1997), and *SNS*, the tetrodotoxin-resistant voltage-gated sodium channel gene (Fig. 3, H vs. G) (Akopian et al. 1996). In contrast to the complete absence of trkA$^+$ neurons in the *ngn1*$^{-/-}$ cervical DRG, in more posterior DRG, a few trkA$^+$ neurons could be detected at E14.5 (not shown). Because some trkA$^+$ cells were seen even in cervical DRG at earlier stages (E12.5), and as development of the posterior trunk DRG lags be-

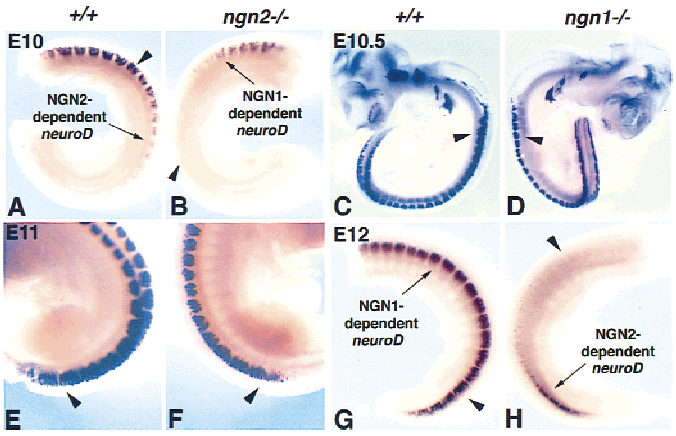

Figure 2. Two transient and overlapping phases of *neuroD* expression are revealed by comparison of *ngn1* and *ngn2* single mutants. All panels show whole-mount in situ hybridization with a *neuroD* probe. Comparison between *ngn2* mutant embryos (*B*,*F*) and wild-type littermates (*A* and *E*, respectively) was performed at E10 (*A*,*B*) or E11 (*E*,*F*). Comparison between *ngn1* mutant embryos (*D*,*H*) and wild-type littermates (*C*,*G*, respectively) was performed at E10.5 (*C*,*D*) or E12 (*G*,*H*). Diminished or no expression of *neuroD* is visible in the caudal region of an E10 *ngn2* mutant embryo (*B*, arrowhead), where earlier-born NGN2-dependent neurons should have been generated (*A*, arrow), whereas it is detectable in the rostral region where later-born NGN1-dependent neurons are beginning to be generated (*B*, arrow). Conversely, diminished or no expression of *neuroD* is visible in the rostral region of an E12 *ngn1* mutant embryo (*H*, arrowhead), where later-born NGN1-dependent neurons would normally be generated at this stage (*G*, arrow), while the last traces of *neuroD* expression are visible in caudal regions (*H*, arrow) where NGN2-dependent neurons are still being generated in these youngest DRG. Note that there is spatial overlap between the NGN2- and NGN1-dependent phases of *neuroD* expression (cf. intensity of staining in *A*, arrowhead vs. *B*; and *G*, arrowhead, vs. *H*). Stages E10 and E12 were chosen to reveal most clearly the NGN1-dependent (*B*, arrow) and NGN2-dependent (*H*, arrow) phases of *neuroD* expression, respectively (see also Fig. 1I, NeuroD). In older *ngn2*$^{-/-}$ embryos (*F*) and younger *ngn1*$^{-/-}$ embryos (*D*), little or no difference in *neuroD* expression is detected in comparison to wild-type embryos (*E* and *C*, respectively). In the case of the older *ngn2* mutant, this is presumably because the later wave of NGN1-dependent *neuroD* expression has already swept into caudal (younger) regions of the embryo (*E* vs. *F*, arrowheads). In the case of the younger *ngn1* mutant it is presumably because the early wave of NGN2-dependent *neuroD* expression has not yet been down-regulated in rostral (older) regions of the embryo (*C* vs. *D*, arrowheads). Note that comparisons of mutant and wild-type littermates from a given stage are presented at the same magnification, but the magnifications of the different stages are not comparable. In *A*, *B*, and *G*, *H* the entire trunk region has been dissected away from the rest of the embryo for clarity. In *E* and *F* only the caudal region of intact embryos is shown.

hind that of the cervical ganglia, it is possible that in *ngn1*$^{-/-}$ embryos some trkA$^+$ neurons or their precursors are transiently generated and then die (see below).

We noted that expression of *ngn1* overlaps the period during which large-diameter neurons are born (Fig. 1I). These neurons, which include mechanoceptive and proprioceptive muscle afferents, express trkB and trkC, which are receptors for brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), respectively (for review, see Chao 1992). Nevertheless, many trkB$^+$ and trkC$^+$ neurons did develop in *ngn1* mutant embryos (Fig. 3I–L); in fact their density appears higher because of the loss of intervening trkA$^+$ cells. However, the second cervical DRG (C2) of E16 embryo *ngn1* mutants is only about a quarter of the size of the wild-type ganglion, and quantification of these neurons in a series of consecutive sections spanning these ganglia revealed a 35% reduction in the total number of trkC$^+$ neurons, and a 27% reduction in the number of trkB$^+$ neurons, in *ngn1* mutants at this stage (Table 1). A 26% reduction in the number of trkC$^+$ neurons in C2 DRG was also observed in *ngn1* mutant embryos at E13 (Table 1), arguing against the idea that *ngn1* is only required for a later-generated subset of trkC$^+$ neurons (Fariñas et al. 1998). We conclude that *ngn1* is required for the development of both a subset of early-generated (trkC$^+$ and trkB$^+$) neurons, as well as for most or all later-generated (trkA$^+$) neurons. TUNEL labeling revealed massive apoptotic cell death in the DRG of *ngn1*$^{-/-}$ embryos at E11.5 (data not shown), suggesting that the fate of many *ngn1*-dependent precursors in the mutant is to die.

Development of the trkC$^+$ and trkB$^+$ neurons is normal in ngn2 *mutants at E13 and later developmental stages*

To determine if the trkC$^+$ and trkB$^+$ neurons spared in *ngn1* mutants were, conversely, dependent on *ngn2*, we examined their development in *ngn2* mutants. Surprisingly, the development of the DRG appeared normal in *ngn2* mutants at E15 and at P0, as indicated by *SCG10* expression (Fig. 4, B vs. A; data not shown). Superficially normal expression was also observed for other markers, including *trkA*, *trkB*, *trkC*, and *ER81* (a marker for a subset of the trkC$^+$ neurons) (Fig. 4C–J) (Lin et al. 1998). To determine if the trkC$^+$ neurons in these mutants make proper projections, we placed DiI crystals into the DRG of newborn wild-type or *ngn2*$^{-/-}$ mutant mice to label the afferents projecting into the spinal cord. The muscle proprioceptive afferents from trkC$^+$ neurons of *ngn2* mutants appeared to send normal projections to the ventral spinal cord (Fig. 4, L vs. K, arrows). To determine if there was a loss of a subset of the trkC$^+$ and trkB$^+$ neurons, we counted these neurons in C2 DRG at two embryonic stages: E16 and E13. No significant loss of either subpopulation was found at E16, and the number of trkC$^+$ neurons was normal at E13 as well (Table 2).

ngn2 *and* ngn1 *together are required for DRG development*

One reason for the apparently normal DRG development observed in *ngn2* mutants might be functional compen-

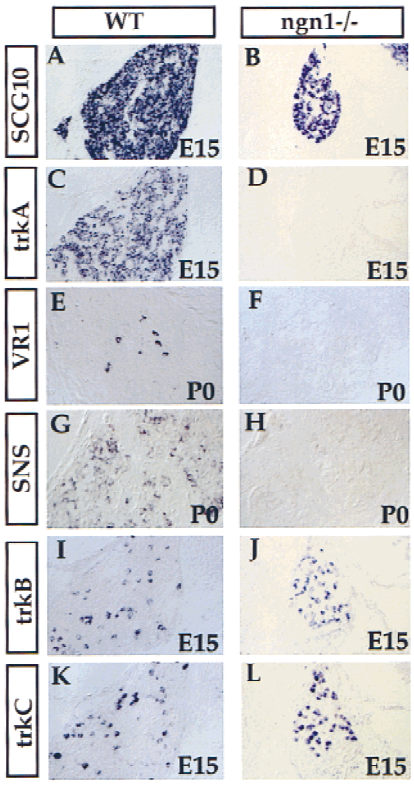

Figure 3. Requirement of NGN1 for the development of the *trkA*$^+$ neurons. Transverse sections through C2 DRG of wild-type and *ngn1* mutant E15 embryos (*A–D,I,J*) or newborn mice (*E–H*) are shown. Section in situ hybridization was performed with the indicated probes.

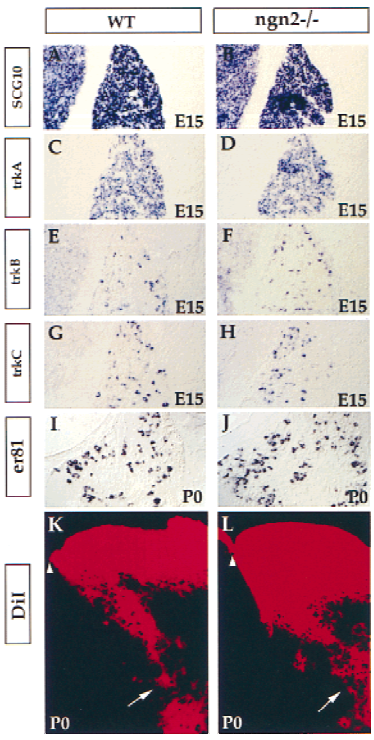

Figure 4. Normal development of the DRG in *ngn2* mutants at E15 and P0. Transverse sections through C2 DRG of wild-type and *ngn2* mutant E15 embryos (*A–H*) or newborn (P0) mice (*I–L*). Section in situ hybridization was performed with the indicated probes. *K* and *L* show DiI labeling of the afferents of the DRG neurons projecting to the neural tube. The projection of proprioceptive muscle afferents to the ventral neural tube appears normal (*K,L*, arrows). Arrowheads indicate the dorsal midline of the spinal cord.

sation by *ngn1*. To examine this possibility, we intercrossed double heterozygous mice to generate *ngn1*$^{-/-}$; *ngn2*$^{-/-}$ double homozygous mutants. DRG were completely absent in E15 double mutant embryos as indicated by the lack of *SCG10* expression (Fig. 5, B vs. A). Identical results were obtained at E12.5 (data not shown). Thus, all DRG neurons require *ngn1* and/or *ngn2*. The apparent lack of a defect in *ngn2*$^{-/-}$ embryos at E13 must reflect redundancy with, or compensation by, *ngn1*.

As *ngn2* is expressed early in neural crest migration (Fig. 1) and some early migrating neural crest cells have been reported to give rise to both sensory and sympathetic neurons (Fraser and Bronner-Fraser 1991), we examined single and double *ngn* mutant embryos for effects on autonomic neuron development. The differentiation of neurons in the sympathetic ganglia was not overtly affected in either of the single or in double *ngn* mutants (Fig. 5C,D; data not shown). Therefore, although *ngns* are required for the development of all DRG sensory neurons, and although *ngn2* is expressed in early migrating neural crest cells, the development of autonomic neurons is unaffected by the lack of these genes.

An early, transient neurogenesis defect in ngn2 *mutants*

The absence of DRG in double mutants (Fig. 5B) implies that the ~75% of trkC$^+$ and trkB$^+$ neurons spared in *ngn1* mutant embryos (Fig. 3; Table 1) must develop from *ngn2*-dependent precursors. It was therefore surprising that we did not detect a loss of the trkC$^+$ and trkB$^+$ neurons in our initial analysis of *ngn2* single mutants (Fig. 4). These observations suggested that because all trkC$^+$

Table 1. *Numbers of trkB*$^-$ *and trkC*$^+$ *neurons in wild-type and* ngn1$^{-/-}$ *embryos*

	E16 C2 DRG			E13 C2 DRG		
	wild type	*ngn1*$^{-/-}$	percent reduction	wild type	*ngn1*$^{-/-}$	percent reduction
trkC	852 ± 56 (4)	554 ± 28 (6)	35% ($P < 0.0005$)	912 ± 9 (4)	675 ± 96 (6)	26% ($P < 0.005$)
trkB	776 ± 53 (4)	563 ± 98 (6)	27% ($P < 0.01$)	N.D.	N.D.	N.D.

Number of ganglia counted in parentheses. (N.D.) Not determined.

Table 2. *Numbers of trkB- and trkC-positive neurons in wild-type and* ngn2$^{-/-}$ *embryos*

	E16 C2 DRG			E13 C2 DRG		
	wild type	*ngn2*$^{-/-}$	percent reduction	wild type	*ngn2*$^{-/-}$	percent reduction
trkC	1041 ± 91 (6)	1153 ± 60 (6)	N.S.	849 ± 62 (6)	885 ± 86 (6)	N.S.
trkB	654 ± 41 (8)	642 ± 112 (6)	N.S.	N.D.	N.D.	N.D.

Number of ganglia counted in parentheses. (N.S.) Not significant. (N.D.) Not determined.

and trkB$^+$ neurons are lost in *ngn1*$^{-/-}$; *ngn2*$^{-/-}$ double mutants, *ngn1* must be compensating in some way for the absence of *ngn2*. To determine if *ngn1* expression was initiated prematurely to substitute for *ngn2*, we examined *ngn1* expression in early *ngn2*$^{-/-}$ embryos. Rather than observing a precocious induction of *ngn1*, we found that the earliest detectable phase of *ngn1* expression was actually lost in *ngn2* mutants (Fig. 6A, arrows). Whereas in wild-type embryos expression of *ngn1* in cervical DRG was first detected at E9–E9.25 (20–21 somites), in *ngn2* mutants it was delayed until E9.5 (24–25 somites) (Fig. 1I, Δt). These data suggest that the earliest expression of *ngn1* in wild-type embryos is NGN2-dependent. Therefore, the apparent compensation by *ngn1* in *ngn2*$^{-/-}$ mutants does not appear to occur by a simple reprogramming of *ngn1* expression to substitute for that of *ngn2*.

These data suggested that compensation by *ngn1* might involve a delay in neurogenesis in *ngn2*$^{-/-}$ mutants. Consistent with this, we observed that *neuroD* expression was initiated substantially later in *ngn2* mutants than in wild-type embryos (see above, Fig. 2B). Moreover, overt neuronal differentiation, as marked by expression of *SCG10*, was also significantly delayed (Fig. 6B). In support of the idea that this delay in neurogenesis reflects the delayed generation of large-diameter neurons, there was a delay in the expression of *trkC* and *trkB* in *ngn2* mutants as well (Fig. 6C,D). Whereas *trkC* could be detected as early as E10 in wild-type embryos (Fig. 6C, left, arrow), no such expression was detected in *ngn2* mutants at this stage (Fig. 6C, right). Rather, *trkC* expression was not detected in *ngn2*$^{-/-}$ embryos until

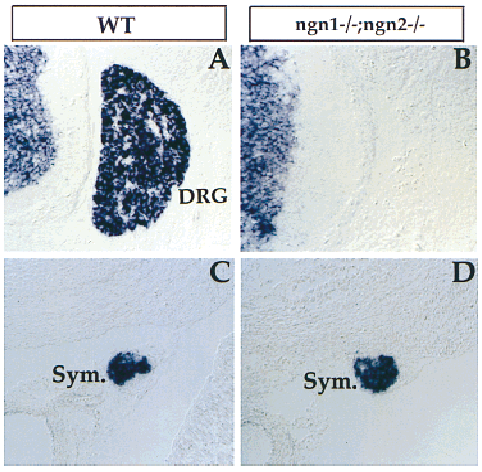

Figure 5. Requirement of NGN1 and NGN2 for DRG development. Transverse sections through an E15 *ngn1* and *ngn2* double homozygous embryo (*B*,*D*) and a wild-type control embryo (*A*,*C*) at lumbar level were probed with *SCG10*, a pan neuronal marker. DRG (*A*, DRG) were absent in double mutants (*B*). Expression of *SCG10* in sympathetic ganglia (*C* and *D*, Sym.) was not affected.

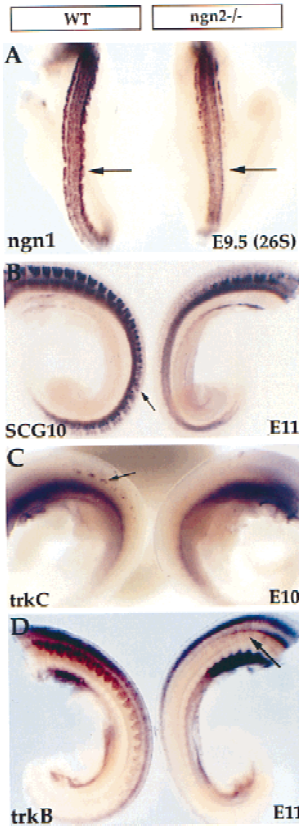

Figure 6. Delayed neurogenesis in *ngn2* mutant embryos. (*A*) E9.5–E9.75 (26S) wild-type and *ngn2* mutant embryos were probed with *ngn1*. Early expression of *ngn1* is lost in the *ngn2* mutant embryo (arrow, *right*). (*B*) Expression of the pan-neuronal marker *SCG10* in caudal DRGs is observed in the wild-type embryo (*B*, *left*, arrow) but is only detected anteriorly in the *ngn2* mutant (*B*, *right*), indicating that neurogenesis is delayed (see also Fig. 2B). (*C*) Initial expression of *trkC* was detected in wild-type embryos at E10 (*left*, arrow) but not in *ngn2* mutant embryos (*right*). (*D*) A delay in the appearance of *trkB*$^+$ neurons is also detected in *ngn2* mutant embryos. In this case, *trkB* expression is not detected until E11 in the mutant (arrow, *right*), consistent with the fact that this mRNA is expressed subsequent to *trkC* mRNA in wild-type embryos as well (not shown).

E10.5–E11 (data not shown). The recovery of *trkB*-expressing neurons occurred even later than that of the *trkC*-expressing neurons (Fig. 6D, right, arrow), consistent with the sequential expression of these two mRNAs in wild-type embryos (data not shown). By E15, the expression of *trkB* and *trkC* in *ngn2*$^{-/-}$ embryos appeared normal (Fig. 4; Table 2). By contrast, no apparent delay was observed in the generation of trkA$^+$ neurons (not shown). Taken together, these data suggest that the development of trkB$^+$ and trkC$^+$ neurons is initially dependent on *ngn2*, but that this dependence is compensated at later stages by *ngn1*.

The delayed neurogenesis in *ngn2*$^{-/-}$ embryos could mean that *ngn2*-dependent precursors wait and are later converted into *ngn1*-expressing cells, which then differentiate into trkC$^+$ and trkB$^+$ neurons. Alternatively, such precursors could die and be replaced by a separate population of *ngn1*-dependent precursors. We therefore examined programmed cell death in wild-type and *ngn2* mutant embryos by the TUNEL method (Gavrieli et al. 1992), at several different stages. Extensive apoptotic cell death was observed at E11 in hindlimb bud-level the DRG of *ngn2* mutant embryos, whereas relatively little cell death was observed in wild-type ganglia at similar axial levels (Fig. 7A,B, arrows). Moreover, on adjacent sections ganglia containing such TUNEL$^+$ cells in mutant embryos did not yet exhibit *SCG10* expression (Fig. 7, C vs. D, arrows), suggesting that many *ngn2*-dependent precursors die before undergoing overt neuronal differentiation. TUNEL$^+$ cells were also observed in more anterior DRG of mutant embryos, at axial levels rostral to the forelimb bud (not shown). However, these cells were observed at earlier stages, consistent with the idea that the requirement for *ngn2* follows the normal rostral-to-caudal progression of DRG development.

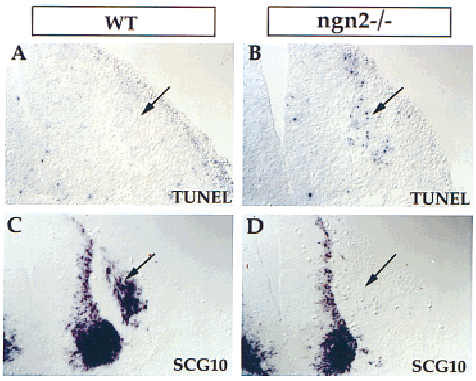

Figure 7. Increased apoptosis precedes overt neurogenesis in *ngn2* mutants. Adjacent transverse sections through E11 wild-type (*A*,*C*) and *ngn2* mutant embryos (*B*,*D*) at an axial level close to the hindlimb bud. Dying cells were detected by the TUNEL method. Extensive cell death was detected in mutant DRG (*B*, arrow) but not in wild-type DRGs (*A*, arrow) at similar axial levels. No *SCG10* expression was detected in an adjacent section through the mutant embryo (*D*, arrow), whereas in wild-type embryos it was already expressed (*C*, arrow).

Discussion

The *ngns* are vertebrate neuronal determination genes that exhibit overlapping expression and apparent functional redundancy in many regions of the CNS. The extent to which this redundancy reflects functional compensation in common or distinct precursors is not clear. We have used the development of the DRG as a model system to address this issue. We find that *ngn1* and *ngn2* are required during different phases of neurogenesis that generate different classes of sensory neurons: *ngn2* is required exclusively during the early phase, whereas *ngn1* is required during the early phase and also exclusively during the late phase. The loss of *ngn2* function, however, appears compensated by *ngn1* after a delay. Many *ngn2*-dependent cells are fated to die in *ngn2*$^{-/-}$ embryos, suggesting that the delayed compensation occurs by substitution of separate *ngn1*-dependent precursors, rather than by reprogramming of *ngn1* expression within *ngn2*-dependent cells.

NGNs and the timing of neurogenesis in the DRG

[^{3}H]Thymidine cell birthdating studies in both mouse (Lawson and Biscoe 1979) and chick (Carr and Simpson 1978) have shown that large- and small-diameter DRG sensory neurons are born in two successive waves. Our observations suggest that these waves may reflect in part the sequential but overlapping utilization of *ngn2* and *ngn1*, respectively (Fig. 8D). This correspondence is not absolute, however, as *ngn1* and *ngn2* are coexpressed during much of the early phase and are both required for the generation of trkB$^+$ and trkC$^+$ sensory neurons. However *ngn1* is exclusively expressed during the later phase and is required for most or all of the trkA$^+$ neurons.

The mechanisms that control the timing of the two waves of neurogenesis are unknown. Despite the absence of *ngn2*-dependent precursors, the generation of *ngn1*-dependent neurons in *ngn2*$^{-/-}$ embryos was still delayed in comparison with wild-type embryos (Fig. 6). This argues against the idea that the delayed differentiation of *ngn1*-dependent precursors is normally controlled by a negative-feedback signal from the earlier-generated *ngn2*-dependent neurons. We cannot exclude the possibility that there is nevertheless some acceleration in the differentiation of *ngn1*-dependent neurons in *ngn2* mutants. However, it seems more likely that the timing of neurogenesis is controlled by the timing of independent inducing mechanisms for *ngn2* and *ngn1*.

The correlation between the utilization of different *ngns* and the generation of different sensory neuron subtypes should not be taken to imply that the NGNs autonomously specify these subtypes, although they could contribute to this specification. In fact, the compensation by *ngn1* observed in the *ngn2*$^{-/-}$ mutant makes it more likely that the NGNs control essentially equivalent programs of sensory neurogenesis. Indeed, we observed a small number of trkA$^+$ neurons in more posterior DRG of *ngn1* mutants, suggesting that some *ngn2*-dependent precursors may contribute to this class of neurons in wild-type animals. If so, then different sen-

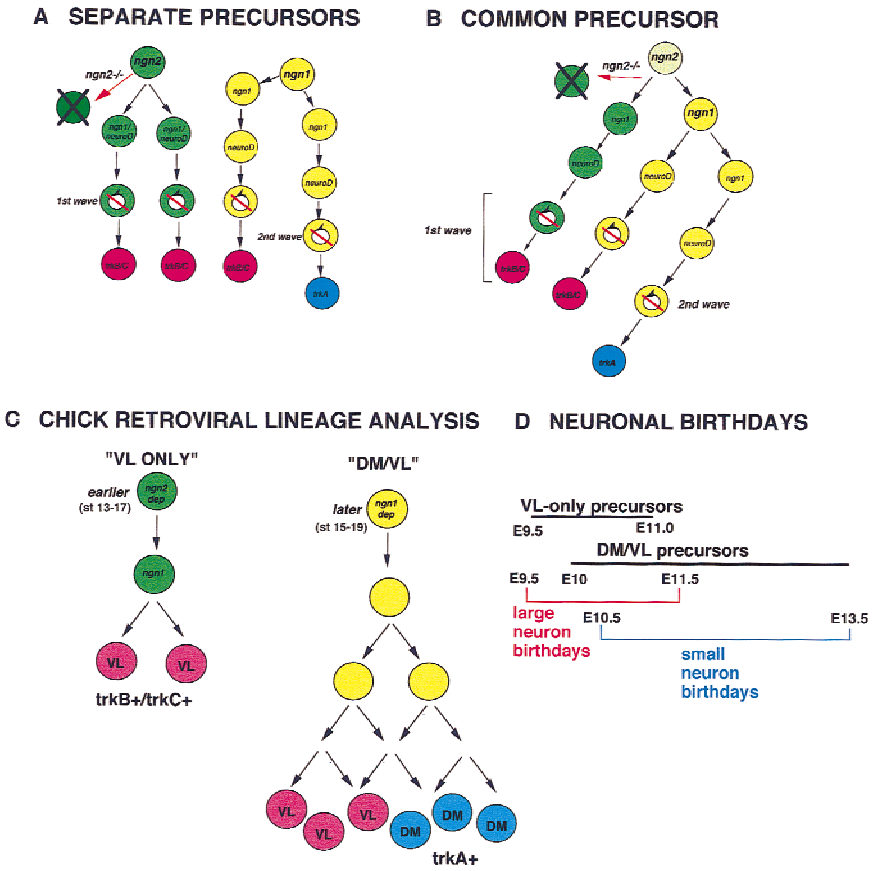

Figure 8. Genetic and lineage relationships between sensory precursors in the DRG. (*A*,*B*) Alternative lineage relationships between *ngn2*- and *ngn1*-dependent precursors. (*A*) *ngn2*-dependent precursors are distinct from *ngn1*-dependent precursors in the DRG. For simplicity, the expression of *ngn1* in the *ngn2*-dependent lineage (see *B*) is shown together with that of NeuroD. (*B*) A common precursor within the DRG that initially expresses *ngn2* generates both *ngn2*-dependent and *ngn1*-dependent precursors. As the early expression of *ngn1* and *neuroD* is lost in *ngn2*$^{-/-}$ mutants (Figs. 2B, 6A), this model requires that such mutant precursors divide to generate some daughters that fail to express *ngn1* or *neuroD* and die (X-ed out cell), and/or other daughters that survive and do express *ngn1* and *neuroD*. Although the model illustrates an asymmetric division of an individual *ngn2*-dependent cell, it is possible that identical cells divide symmetrically to generate either two daughters that survive, or two daughters that die. Slashed circular arrows indicate cell cycle withdrawal. (*C*) Correlation of genetic dependence on *ngn2* or *ngn1* with different sensory neuron precursor populations identified by retroviral lineage tracing in chick (Frank and Sanes 1991; and unpubl.). (Earlier; later) The embryonic stages (Hamburger and Hamilton 1951) at which such precursors are observed. VL neurons (red circles) located in the ventrolateral region of the DRG and correspond to large-diameter trkB$^+$ and/or trkC$^+$ neurons; DM neurons (blue circles) are located in the dorsomedial region of the DRG and correspond to small-diameter trkA$^+$ neurons. (*D*) Time line emphasizing that the birthdays of large-diameter neurons in the DRG (Lawson and Biscoe 1979) likely are initiated by VL-only (*ngn2*-dependent) precursors but extend into the period when DM/VL (*ngn1*-dependent) precursors are also giving rise to trkB$^+$ and/or trkC$^+$ (VL) neurons. We cannot exclude that some VL-only precursors are *ngn1*-dependent. Small-diameter neurons are produced primarily or exclusively by DM/VL precursors, which are *ngn1*-dependent.

sory neuron subtypes are likely determined by factors other than the *ngns*. The action of such factors may nevertheless require NEUROGENIN function. In that case, the sequential utilization of *ngn2* and *ngn1* could simply control the timing of neurogenesis, with the action of subtype-determining factors dependent on this timing (Tanabe 1998; Edlund 1999).

Do NGN2-dependent cells constitute a distinct subpopulation of sensory precursors?

The fact that different subclasses of sensory neurons are born on different schedules raises the possibility that they may be generated from distinct subsets of neural crest-derived precursors (Fig. 8A,B). Alternatively, a common precursor within the DRG could divide asymmetrically to sequentially generate early- and late-born neurons (Fig. 8A,B). Our data favor (but do not prove) the former possibility. In *ngn2*$^{-/-}$ embryos, extensive apoptosis is observed in ganglia that have not yet expressed *SCG10* (Fig. 7B,D), and therefore likely reflects the death of *ngn2*-dependent precursor cells. However, these ganglia already exhibit *neuroD* expression on adjacent sections (Q. Ma, unpubl.). As *neuroD* expression is initially prevented in *ngn2*$^{-/-}$ embryos (Fig. 2B), the subsequent expression of *neuroD* in the face of extensive cell death must occur in surviving precursors that have already expressed *ngn1*. For such surviving cells and those killed by the *ngn2* mutation to derive from the same progenitor, it is necessary to postulate that this progenitor divides to generate both a daughter cell that fails to express *ngn1* (or *neuroD*) and dies (Fig. 8B, *ngn2*$^{-/-}$), and a daughter that survives to express *ngn1* and *neuroD* and that differentiates. Although such an explanation is formally possible, it seems simpler to think that *ngn2*-dependent and *ngn1*-dependent precursors represent distinct lineages in the DRG (Fig. 8A).

Interestingly, evidence for the existence of two types of sensory precursors has been provided by a retroviral lineage analysis of avian DRG development (Frank and Sanes 1991). In the chick, large-diameter trkC$^+$ (and some trkB$^+$) neurons tend to be located in the ventrolateral (VL) region of the DRG, whereas small-diameter trkA$^+$ neurons tend to be located in the dorsomedial

(DM) region (Carr and Simpson 1978; Kahane and Kalcheim 1994; Wright and Snider 1995; Backstrom et al. 1996). Two types of retrovirally marked clones containing neurons were obtained in this experiment. One, which is small (3 cells/clone), contained exclusively large diameter VL neurons; the other, which is large (~35 cells/clone), contained both small diameter DM neurons and larger VL neurons (Fig. 8C). Interestingly, VL-only clones were encountered only when viral infection was performed at earlier stages (Hamilton and Hamburger 1951; stage 13–16); whereas DM/VL clones were more frequent when infection was carried out later (stage 17) and were rare for infections made before stage 15 (E. Frank, pers. comm.). This observation is opposite to what one would expect if VL-only clones simply represented more restricted progeny of DM/VL precursors. Furthermore, DM/VL clones were large even when obtained with earlier (before stage 17) injections, whereas VL-only clones in the same embryos were small. As retroviral infection occurred within the neural tube, prior to emigration, these data strongly suggest the existence of a distinct, early precursor population with limited proliferative capacity that generates only large-diameter neurons. The sequential but overlapping waves of neuronal birthdays observed in thymidine-labeling experiments (Lawson and Biscoe 1979) would then reflect the fact that VL-only precursors are generated first, followed by a period of overlap with DM/VL precursors, followed by a period when only DM/VL precursors are observed (Frank and Sanes 1991; E. Frank, pers. comm.; see Fig. 8C,D).

These results are remarkably congruent with our observation that *ngn2* is required only for the development of early-generated trkC$^+$ and trkB$^+$ neurons, whereas *ngn1* is required for most or all later-generated trkA$^+$ neurons (as well as for a subset of trkC$^+$ and trkB$^+$ neurons). This correlation suggests that most VL-only precursors likely correspond to *ngn2*-dependent precursors (and perhaps to a subset of *ngn1*-dependent precursors), whereas most DM/VL precursors likely correspond to *ngn1*-dependent cells. We emphasize that this correspondence is based on the differential genetic requirement for either *ngn2* or *ngn1*, not on mere expression of these genes that likely can occur in both lineages. Thus, for example, the initial phase of *ngn1* expression is *ngn2*-dependent, and likely occurs in VL-only precursors (Fig. 8C, VL only), whereas the later, *ngn2*-independent phase of *ngn1* expression likely reflects expression in DM/VL precursors. This interpretation is supported by the observation that within developing cranial sensory ganglia, NGN2 activates *ngn1* expression in epibranchial placode-derived neuronal precursors, whereas in trigeminal placode-derived precursors, *ngn1* expression is independent of NGN2, but conversely activates *ngn2* expression (Fode et al. 1998; Ma et al. 1998). These data argue that expression of the *ngns* can be both independently regulated in distinct sensory lineages, as well as cross-regulated within a given lineage. Consistent with this, preliminary analysis of *ngn2–lacZ* knock-in mice suggests that most or all sensory neurons express *ngn2* at some point in their developmental history (Q. Ma, C. Fode, F. Guillemot, and D. Anderson, unpubl.). Whether this reflects an early, transient expression of *ngn2* in DM/VL precursors, or cross-regulation of *ngn2* by *ngn1* at a later stage, is currently being investigated. Whatever the case, the results emphasize that expression patterns of the *ngns* alone do not predict lineage relationships in the DRG.

The association of *ngn1*- and *ngn2*-dependency with distinct types of sensory precursors would also be consistent with the observation that VL-only clones are smaller than DM/VL clones (Frank and Sanes 1991; Fig. 8C). Expression of *ngns* leads to expression of *neuroD* (Ma et al. 1996), which in turn may promote cell-cycle withdrawal (Morrow et al. 1999). Cells that already express *ngn2* early in crest migration (Fig. 1) might therefore undergo relatively few divisions after arriving at the DRG. This would explain why VL-only clones are small. Conversely, as most *ngn1* expression occurs after ganglion condensation, *ngn1*-dependent precursors would have more time to divide between the time they emigrated from the neural tube and the time they expressed *neuroD*. Consequently, DM/VL clones would be relatively larger, as observed in the chick studies.

Why have two separate lineages of sensory precursors? The fact that *ngn2* is expressed early in neural crest migration suggests that the VL-only precursors that require its function may be specified for a sensory fate shortly after they emigrate from the neural tube. Consistent with this idea, forced expression of *ngn2* in premigratory chick neural crest cells can bias them to a sensory fate (Perez et al. 1999). Furthermore, *ngn2*-expressing precursors in neural crest explant cultures appear committed to a sensory neuron fate in the presence of the autonomic-inducing signal, BMP2 (Greenwood and Anderson 1999). If VL-only precursors are determined for a sensory fate, they could provide a population of pioneer neurons to seed the developing DRG, and form a cellular scaffold around which to assemble the other neuronal subtypes that develop in these ganglia. Analagous pioneer precursors have been described in muscle development (Kahane et al. 1998), and in this respect it is interesting that the sensory pioneer neurons are those that innervate muscle.

Possible compensation mechanisms in ngn2 *mutants*

If the *ngn1*-dependent (DM/VL) precursor population has the capacity to generate a larger number of trkC$^+$ and trkB$^+$ neurons than it is normally required to, then in wild-type embryos *ngn2*-dependent (VL-only) precursors (or their progeny) must limit the number of such neurons generated from the former *ngn1*-dependent precursor pool. There are several possible mechanisms by which this could occur. One is by competition for limiting amounts of neurotrophic factors. Perhaps surplus neurons generated from the *ngn1*-dependent precursors that normally die are rescued to provide a full complement of neurons in the *ngn2* mutant. Alternatively, production of trkB$^+$ and trkC$^+$ neurons may ultimately be limited by

a negative-feedback signal, threshhold levels of which are normally reached before *ngn1*-dependent precursors are able to generate >25%–30% of all these neurons. In *ngn2* mutants release from such feedback inhibition would allow the production of extra trkB$^+$ and trkC$^+$ neurons from *ngn1*-dependent precursors. It is important to emphasize that this negative feedback is postulated to control the number of trkB$^+$ and trkC$^+$ neurons that are generated, not the time at which they are produced (see above). There is precedent for such feedback inhibition of neuronal subtype generation in other systems (Reh 1986; Belliveau 1999; Belliveau and Cepko 1999).

Cellular mechanisms underlying apparent genetic redundancy

Our observations for *ngn1* and *ngn2* show remarkable parallels to those made previously for the myogenic determination factors *myf5* and *MyoD*. During somitic development, *myf5* is expressed initially by the earliest myogenic precursors at the DM edge of the myotome that give rise to epaxial (deep back) muscle, whereas *MyoD* is expressed later-differentiating hypaxial precursors located in the VL myotome (for reviews, see Molkentin and Olson 1996; Yun and Wold 1996; Tajbakhsh and Cossu 1997). The idea that *myf5* and *MyoD* are expressed initially by distinct muscle lineages is also supported by in vitro studies (Braun and Arnold 1996). These results provide a precedent for the idea that *ngn2* and *ngn1* may define distinct populations of sensory precursors in the DRG, although this remains to be formally proven. Later in development, cross-regulation by the MRFs occurs in both myogenic sublineages (for review, see Tajbakhsh and Cossu 1997). Similarly, cross-regulation by the *ngns* can occur in distinct sensory sublineages (Fode et al. 1998; Ma et al. 1998); a subtle difference is that in the DRG, cross-regulation of *ngn1* by NGN2 appears to occur in the VL-only lineage before *ngn1* is activated independently in the DM/VL lineage.

Striking parallels are also seen in the compensation phenomena observed in single mutants. In *myf5* null mice, the development of epaxial muscle is initially blocked, but is recovered after a delay in a *MyoD*-dependent manner (Braun et al. 1992, 1994; Tajbakhsh et al. 1996). This is remarkably analogous to the delayed recovery of trkB$^+$ and trkC$^+$ neurons in *ngn2*$^{-/-}$ mice. In the case of muscle, controversy still exists regarding the question of whether such compensation reflects the substitution of *MyoD*-dependent precursors for *myf5*-dependent precursors (Patapoutian et al. 1995; Braun and Arnold 1996; Molkentin and Olson 1996; Yun and Wold 1996), or rather a delayed activation of *MyoD* expression in the normally *myf5*-dependent lineage (Tajbakhsh and Cossu 1997; Tajbakhsh et al. 1998). As discussed earlier, our apoptosis data support the idea of cellular compensation in *ngn2*$^{-/-}$ mice. However, we cannot exclude the possibility completely that only a subset of *ngn2*-dependent precursors die in *ngn2*$^{-/-}$ mice, and that the rest wait and then go on to express *ngn1*. An apparent difference between the muscle and neural systems is that no obvious muscle defects are seen in either *myf5* or *MyoD* single mutants, whereas in *ngn1* single mutants the trkA$^+$ population is almost completely eliminated. As a few trkA$^+$ cells are found in *ngn1*$^{-/-}$ embryos and therefore presumably can be produced by precursors that express *ngn2*, we think that the inability of the *ngn2*-dependent VL-only lineage to compensate for the loss of trkA$^+$ neurons in *ngn1* mutants most likely reflects its limited proliferative capacity (Frank and Sanes 1991), rather than an inherent functional difference between the NGNs.

The initial expression of *myf5* and *MyoD* in epaxial and hypaxial myoblasts is induced by distinct signals produced by different tissues: the neural tube and dorsal ectoderm, respectively (for reviews, see Cossu et al. 1996; Tajbakhsh and Cossu 1997). Although little is known about the nature of the signals that control the initial expression of *ngn2* and *ngn1*, by analogy to muscle it seems likely that they are induced by distinct factors, or by related factors produced by different tissues at different times. Moreover, the fact that the DRG develop in close physical association with the somites, taken together with the striking parallels in the expression and function of myogenic and neurogenic bHLH factors, raises the possibility that the induction of the *ngns* is controlled by analogous, if not homologous, signaling mechanisms. The identification of these signals should help clarify the mechanisms that control the timing of neurogenesis in the DRG.

Materials and methods

Animals

The generation of *ngn1* and *ngn2* mutant mice has been described previously (Fode et al. 1998; Ma et al. 1998). Single heterozygous or double heterozygous mice were intercrossed to generate homozygous embryos. The morning that vaginal plugs were observed has been considered as E0.5. Embryos at E10, E11, and E12 were collected in PBS, fixed in 4% paraformaldehyde overnight, and stored in methanol for whole-mount in situ hybridization. Embryos at E13, E15, and E16 were fixed overnight in 4% paraformaldehyde, sunk in 30% sucrose, and embedded in OCT (Tissue-Tek) for cryostat sectioning. Newborn mice were perfused with PBS followed by 4% paraformaldehyde, immersed overnight in 4% paraformaldehyde, sunk in 15% sucrose, and embedded in OCT for sectioning.

In situ hybridization

Section and whole-mount in situ hybridization were performed as described previously (Ma et al. 1998). Detailed protocols are available upon request. Probes used in this study include *ngn1* (Ma et al. 1998), *ngn2* (Fode et al. 1998), rat *SCG10*, probe *SCG10-8* (Stein et al. 1988), mouse *neuroD* (Lee et al. 1995), *trkA* and *trkB* (Birren et al. 1993), *trkC* (kinase-domain-containing probe, from Mariano Barbacid), *er81* (Lin et al. 1998), and *Hfh2* (Labosky and Kaestner 1998).

TUNEL staining on cryostat sections

Frozen sections were collected on Super-Frost/plus microscope slides (Fisher), dried at 37°C for 20 min, fixed in 4% paraformaldehyde for 30 min at room temperature, and washed twice with PBS for 10 min each. Slides were then incubated in per-

meabiliation solution (0.1% Triton X-100 plus 0.1% sodium citrate) for 10 min on ice, rinsed twice with PBS, incubated with 200 µl of 3% H_2O_2 with coverslips for 15 min, preincubated with 100 µl TDT (terminal deoxynucleotide transferase, Boehringer Mannheim) buffer (30 mM Tris at pH 7.2, 140 mM sodium cacodylate, 1 mM $CoCl_2$, 0.5 mM DTT) with coverslips for several minutes, incubated with a 100 µl of reaction mixture (30 mM Tris at pH 7.2, 140 mM sodium cacodylate, 1 mM $CoCl_2$, 0.5 mM DTT, 10 µM biotin–dUTP, 0.3 U/µl TDT) for 1 hr at 37°C in a humidified chamber, and washed three times with PBS for 5 min each. The Vectastain ABC kit was used to detect the biotin-labeled DNA.

Cell counts

Sections from E13 and E16 embryos were used. Three sets of 10-µm transverse sections through C2 DRG were collected and probed with *trkC*, *trkB*, and *trkA*, respectively. Nuclear-containing positive cells were counted bilaterally. Numbers were not corrected.

DiI tracing

Newborn mice were fixed by perfusion with 4% paraformaldehyde. DiI crystals were inserted into lumbar/thoracic DRG. Mice were then incubated in 4% paraformaldehyde for 2 weeks at 37°C, sunk in 15% sucrose overnight, and embedded in OCT. Transverse sections (30 µm) were collected and viewed using a Bio-Rad MRC600 confocal microscope.

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